Developmental Cell
Perspective

Quantitative Analysis of Embryogenesis: A Perspective for Light Sheet Microscopy

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It is a challenge in developmental biology to understand how an embryo's genes, proteins, and cells function and interact to govern morphogenesis, cell fate specification, and patterning. These processes span very different spatial and temporal scales. Despite much progress, simultaneous observation of such vastly differing scales has been beyond the scope of conventional microscopy. Light sheet microscopy fills this gap and is increasingly used for long-term, high-speed recordings of large specimens with high contrast and up to subcellular spatial resolution. We provide an overview of applications of light sheet microscopy in developmental biology and discuss future perspectives in this field.

Light Sheet Microscopy Bridges Spatial and Temporal Scales during Embryonic Development

Imaging is established as one of the most powerful techniques in developmental biology because it permits minimally invasive observations in space and time, a fundamental requirement to examine living processes. Fluorescent proteins and dyes have enabled applications from cell and tissue lineage tracing to the visualization of molecular details. In fluorescence microscopy, the generation of image contrast, i.e., a sufficiently high signal-to-noise ratio, requires acquisition time and thus collides with the spatiotemporal sampling needed to image small objects such as cell nuclei (~5 μm diameter) within a large specimen like the zebrafish embryo (*Danio rerio*, ~1 mm diameter). Resolving the nuclei or even smaller structures requires high spatial sampling, but to densely cover the entire embryo, a vast number of points must be recorded. The resulting difficulties may be best illustrated in a rough estimation: If 1 mm^3 containing a zebrafish embryo should be covered by an xy-sampling of 1,024 square pixels and a three times courser z-sampling, 358 million points must be acquired. Using photomultiplier tube (PMT) detectors, a measurement time of 3 μs is realistic to generate sufficient signal. In conventional confocal and two-photon microscopy, this measurement time must be applied to each point individually (the pixel dwell time). Acquiring this volume therefore takes around 20 min, during which the embryo continues to develop. Zebrafish cleavage divisions, for instance, occur roughly at the same rate, resulting in a doubling of cell number during imaging from the first to the final point of the volume. In contrast, the temporal sampling required for cell tracking in zebrafish embryos has been estimated to be 60 s or higher (Khairy and Keller, 2011). Achieving sufficiently fast point scanning comes at the price of a devastating reduction of spatial sampling and/or image contrast. To solve this problem, microscopy techniques such as spinning disc microscopy (Gräf et al., 2005) parallelize pixel measurements. However, as confocal and spinning disc microscopes iterate over the z-planes of a three-dimensional (3D) sample, all z-planes are illuminated each time a single plane is recorded. Light sheet fluorescence microscopy (LSFM) takes a different approach by selectively illuminating and capturing only the current focal plane. Furthermore, in contrast to point scanning, the entire plane is illuminated at once, and using sensitive high-speed cameras, 20 and more densely sampled planes can be acquired in a single second. Tomer et al. (2012) report a sustained recording rate of 175 million voxels/s and achieve sufficient temporal resolution and contrast to facilitate automated cell tracking in fruit fly (*Drosophila melanogaster*) embryos.

Light sheet microscopy dates back to 1903 (Siedentopf and Zsigmondy, 1903), but it was Voie et al. (1993) who first applied it to biological samples. Its breakthrough in developmental biology occurred when long-term live imaging of medaka fish (*Oryzias latipes*) and *Drosophila* embryos was accomplished (Huisken et al., 2004), followed by the detailed 3D time-lapse (3D+t) recording of early zebrafish morphogenesis in toto (Keller et al., 2008). The prevalent LSFM designs used in developmental biology (e.g., Figure 1A) feature a stage that moves the sample through a thin sheet of laser light (Figure 1B). This light sheet is arranged perpendicular to the detection objective and illuminates the entire focal plane (Figures 1C and 1D). Since fluorescence is only generated within the illuminated volume, optical sectioning is inherent. As indicated earlier, the entire illuminated plane is recorded using sensitive high-speed charge-coupled device (CCD) and complementary metal oxide semiconductor (sCMOS) cameras. Importantly, while the sample is moved through the laser sheet during the recording procedure, only a small part is exposed at any given time. Phototoxicity and bleaching are thereby reduced by several orders of magnitude (Keller et al., 2008), which is a vital requirement for the long-term recording of live specimens.

Essentially every imaging technique is subject to light absorption and scattering, resulting in uneven illumination and successively decreasing signal and resolution at higher penetration depth. A limitation of most LSFM designs is that they are wide-field microscopes that rely on the optical sectioning intrinsic to sheet illumination. For this, they are sensitive to absorption and scattering artifacts that manifest across the illuminated xy-plane (Figure 1E). This general problem and the specific requirements of the research conducted in several labs have driven a diversification of the technology and continue to motivate incremental improvements. Some key accomplishments in this rapidly

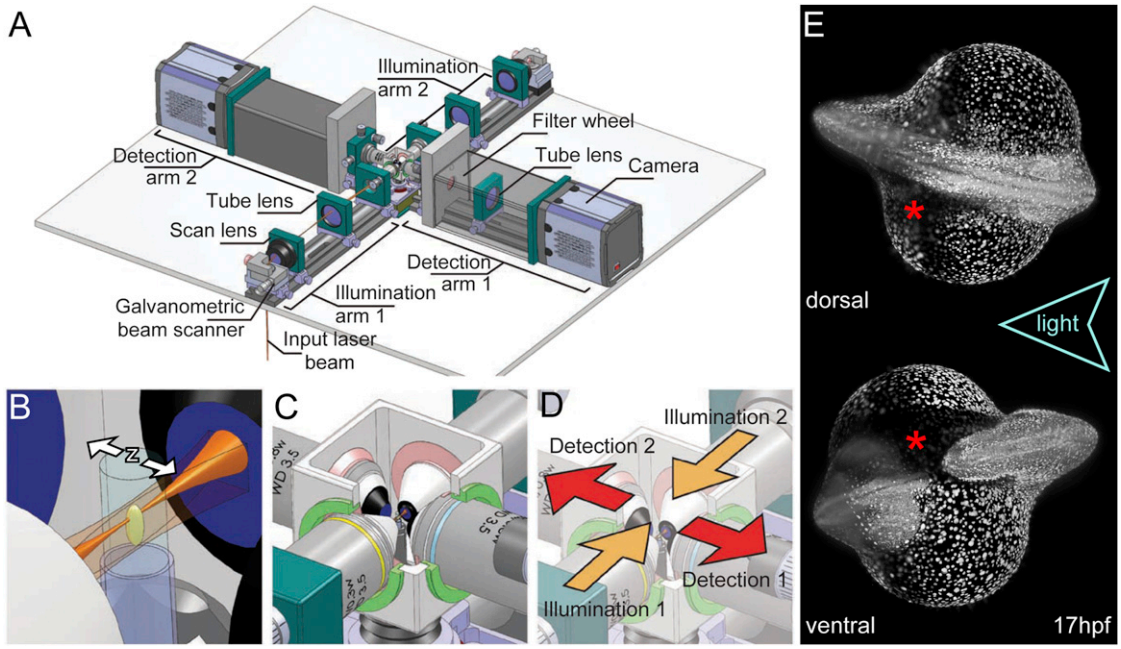

Figure 1. Light Sheet Fluorescence Microscopy

(A–D) Light sheet fluorescence microscope (LSFM) setup. The basic LSFM has two optical arms, one for detection (Detection arm 1) and one for illumination (Illumination arm 1), arranged perpendicular to each other. (A) Bidirectional illumination and detection (Detection arms 1 and 2, Illumination arms 1 and 2) improves illumination of large and scattering samples. In contrast to unidirectional illumination/detection, a bidirectional setup enables in toto observation without sample rotation. (B) Specimen embedded in agarose cylinder within sample chamber, moving through the xy-oriented light sheet (in z-direction). (C and D) Sample chamber with four-way optical access permitting bidirectional illumination and rotation-free multiview acquisition. Adapted from Krzic et al. (2012), with permission from Macmillan Publishers Ltd.

(E) Maximum intensity projection of two opposite views of a zebrafish ubiquitously expressing histone-EGFP and imaged with LSFM (17 hr postfertilization [17 hpf]). Since after the onset of gastrulation, the embryo developed under the microscope despite being imaged in toto every 2 min. In this case, the unidirectional illumination (arrowhead) resulted in signal degradation (red asterisks) that is due to scattering, refraction, and absorption. The two strategies to overcome this problem are bidirectional illumination, shown in (D), and multiview acquisition (see main text).

moving field are the use of bidirectional illumination with pivoting light sheets (Huisken and Stainier, 2007); scanned light sheets (Keller et al., 2008); incoherent structured illumination (Keller et al., 2010); two-photon light sheets, both static (Palero et al., 2010) and scanned (Planchon et al., 2011; Truong et al., 2011); and the use of single- and two-photon self-reconstructing bessel beams (Fahrbach et al., 2010; Planchon et al., 2011). Approaches to combine LSFM and confocal detection (Kalchmair et al., 2010; Fahrbach and Rohrbach, 2012), adaptive optics (Turaga and Holy, 2010), and stimulated emission depletion (STED, Friedrich et al., 2011) have been reported as well. A complete list and detailed description of the different LSFM implementations and their underlying strategies has been compiled elsewhere (Huisken and Stainier, 2009; Huisken, 2012; Keller and Dodt, 2012; Khairy and Keller, 2011; Reynaud et al., 2008; Santi, 2011; Tomer et al., 2011; Weber and Huisken, 2011).

An approach widely used across the different instrumentation designs is multiview acquisition, whereby the sample is recorded from multiple directions (Shaw, 1990; Huisken et al., 2004; Swoger et al., 2007). The individual stacks are then computationally merged into a single volume with maximal data quality (Preibisch et al., 2010; Rubio-Guivernau et al., 2012; Temerinac-Ott et al., 2012), which achieves a more isotropic overall resolution and increases coverage of specimens that are too large or opaque to be imaged from a single direction only. Until recently, multiview acquisition was accomplished by rotating the sample, which tends to be too slow for the desired time intervals of some applications and necessitated time-intensive data postprocessing. Tomer et al. (2012) and Krzic et al. (2012) (Figures 1A–1D) overcame this limitation by placing two cameras on opposite sides of the specimen. A combination with bidirectional illumination permits the recording of entire samples without rotation.

Taken together, the acquisition speed of LSFM enables imaging large specimens such as entire zebrafish embryos with sufficient resolution and sampling to resolve comparatively small structures like cell nuclei. Importantly, the high recording speed is combined with minimized photoexposure, which opens the opportunity to achieve high temporal sampling over extended time periods. This feature combination is unavailable with conventional imaging techniques and makes LSFM particularly well suited to perform system-wide observations and interrogations of embryogenesis.

Tracking Morphogenesis and Mitotic Lineages

The observation of tissue and organ formation at the cellular level is a fundamental step to understand development. The ideal description of a morphogenetic event provides detailed time-resolved information on the behavior of all involved individual cells over its entire duration. This aim is hard to fulfill in practice,

and the challenges vary for different model organisms. Opacity, size, speed of development, cell number and density, cell mixing, and division rates are some of the most important considerations in this context. In addition, the long-term imaging capabilities of LSFM can only be fully exploited if the specimen is permitted to develop and grow under physiological conditions. One particularly important aspect is the specimen mounting, which differs substantially from the strategies usually used in confocal microscopy (e.g., Figure 1B). To observe zebrafish embryogenesis, for instance, it is important to balance the immobilization needed to facilitate imaging and the requirements of the embryo to take shape and grow. If the embryo cannot be recorded within its chorion, very soft embedding within a rigid tube made of a material with a refractive index close to that of water has recently been used as a solution (Kaufmann et al., 2012). The requirements for other organisms like the plant *Arabidopsis thaliana* are quite different. Maizel et al. (2011) generated near-natural conditions in the sample chamber of their LSFM by permitting vertical growth, with the root embedded in a gel cylinder and leaves left free in the air. A cold light source additionally allowed the emulation of day–night cycles by optionally illuminating the plant between recording intervals. It will be interesting to see whether similarly well-designed solutions can be developed for organisms with inherently high embryo culture requirements such as mouse (*Mus musculus*; Udan and Dickinson, 2010).

With respect to morphogenesis and, in particular, mitotic lineage, the highest level of understanding has so far been achieved for invertebrates, of which many have invariant lineages. The best understood metazoan species in this context is the nematode *Caenorhabditis elegans*. Its transparency and small size enabled the sustained effort leading to the elucidation of its entire invariant lineage using light microscopy and Nomarski optics (Sulston and Horvitz, 1977; Sulston et al., 1983). Video microscopy could only be applied during early *C. elegans* development, until the diameter of its body became too thick and 3D cell divisions had to be worked out by manually focusing through the specimen. Today, such undertakings are supported by sophisticated 3D+t microscopy and fluorescent markers (e.g., Schnabel et al., 1997; Bao et al., 2006). Software packages furthermore allow the manual assignment of cell positions and divisions while interactively browsing the data in time and space (Schnabel et al., 1997; Eliceiri et al., 2012) or even provide algorithms that largely automate this task (Bao et al., 2006; Boyle et al., 2006; Murray et al., 2006; Dzyubachyk et al., 2009) up to automatic assignment of cell identity (Murray et al., 2008; Long et al., 2009; Qu et al., 2011).

Higher invertebrate and vertebrate models such as *Drosophila*, zebrafish, and mouse are less amenable to lineaging by direct observation. In contrast to *C. elegans*, their lineage is regulative, and cell migration and mixing are much more prevalent during development. For this, observing individual cell behavior and the establishment of a mitotic lineage requires the tracking of cell movements over time. This approach has produced detailed insights into, e.g., mesoderm migration during *Drosophila* gastrulation, the behavior of retinal progenitor cells, and the zebrafish mitotic lineage up to midblastula transition (McMahon et al., 2010; Rembold et al., 2006; Olivier et al., 2010). These studies focused on the observation of relatively local events or short time periods, which is due in part to the technical limitations of point scanning. LSFM can be used to circumvent this caveat. For instance, Keller et al. (2008) used LSFM and automated image processing to generate the first comprehensive data set of cell positions during early zebrafish embryogenesis (Figure 2A). The high recording rate of LSFM was furthermore used to image morphogenesis of the intact, beating zebrafish heart (Scherz et al., 2008). Within just a few years, particularly astonishing progress has been achieved for in toto imaging of *Drosophila* development. Keller et al. (2010) used this model to demonstrate the image contrast enhancement achieved by a light sheet-based implementation of incoherent structured illumination. Truong et al. (2011) utilized two-photon scanned LSFM to cover the entire fly development with excellent penetration depth. Very recently, long-term in toto imaging at 30 s time intervals has been achieved with scanned single-photon (Krzic et al., 2012) and scanned two-photon LSFM (Tomer et al., 2012), covering essentially the entire embryonic development. Both studies additionally perform automated cell tracking during syncytial blastoderm stages, demonstrating the potential of this technique for the retrospective analysis of cell behavior and lineage. Notably, these four manuscripts were published within a period of only 2 years, and each introduced novel microscopy technology. This illustrates the high pace at which the field develops. The data quality in these publications is unprecedented, and the movies in their online supplements are highly recommended.

Data with high temporal sampling contains valuable information about cell behavior and enables a retrospective analysis. To this end, cells of interest are selected in their differentiated state and final position and are computationally traced backward through the time lapse. This enables systematic investigations into the behavior of cells with a known common fate without requiring a preconception of their origin. Swoger et al. (2011) performed a retrospective analysis of the posterior lateral line of zebrafish. Unlike the ubiquitous chromatin label used by Keller et al. (2008), they made use of tissue and cell type specific labeling to focus on the small cell population of interest and a specific cell type therein. As one of the labels was membrane-localized, their data include additional information on cell morphology. Intended as a small-scope, proof-of-concept study using manual cell tracking, this analysis is not comprehensive, yet the authors re-emphasize the fidelity of LSFM for retrospective cell lineage analysis and compare it directly and favorably to spinning disc microscopy.

Manual tracking as in Swoger et al. (2011) is very accurate but does not scale well with increasing cell counts and time periods. Hence, it is desirable to automate this task. Tomer et al. (2012) and Krzic et al. (2012) accomplished fully automated in toto cell tracking during *Drosophila* syncytial blastoderm stages with high accuracy. However, this impressive accomplishment must be interpreted considering that a single error may affect multiple lineages and is propagated to all subsequent time points. The accuracy of cell tracking, both manual and automated, will therefore successively degrade over time, which raises fundamental scalability concerns. One way to counterbalance this problem could be to combine automated cell tracking with clonal labeling techniques. This may reduce ambiguities when matching cells across time points and could

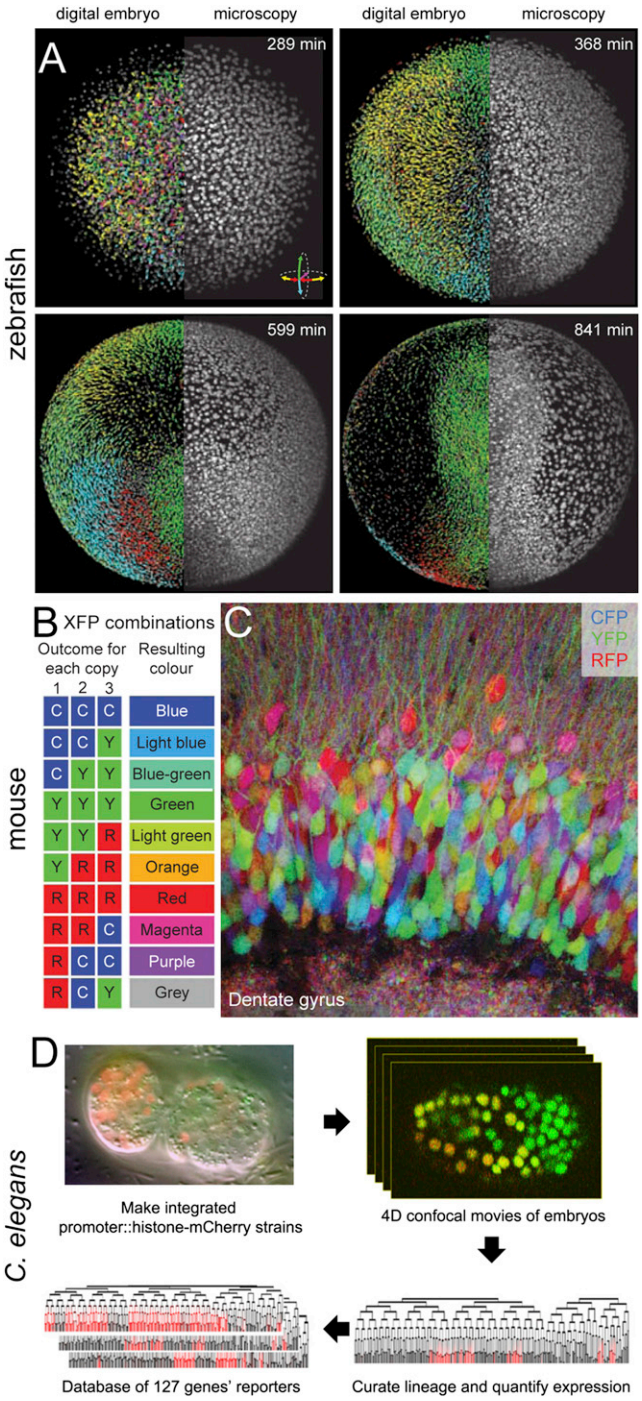

Figure 2. Tracking Morphogenesis and Mitotic Lineage

(A) Computational in toto cell tracking provides a digital representation of zebrafish morphogenesis (digital embryo). Microscopy data (right half of embryo: animal view, maximum projection) and digital embryo (left half of embryo) with color-coded migration directions. Color code: cyan, dorsal migration; green, ventral migration; red or yellow, toward or away, respectively, from body axis; pink, toward yolk. Reprinted from Keller et al. (2008) with permission from the American Association for the Advancement of Science (AAAS).

(B and C) Combinatorial Brainbow with three multiple genomic insertions extends the color palette. (B) Possible recombination outcomes result in discernible fluorescent protein (XFP) color combinations. (C) Stochastically labeled neurons in the mouse dentate gyrus. Adapted from Livet et al. (2007) with permission from Macmillan Publishers Ltd.

(D) Cell and lineage-resolved quantitative gene expression readout in *C. elegans*. Confocal movies were analyzed by automated cell recognition to generate a cell lineage tree. Reporter expression (histone-mCherry) was visualized by converting the raw reporter intensity in each cell into a color on a scale from black to red (from minimum to maximum expression) and displaying the color on the appropriate branch of the lineage tree. Reprinted from Murray et al. (2012) with permission from Cold Spring Harbor Laboratory Press.

provide direct biological ground truths to measure tracking fidelity.

In clonal analysis, individual cells are unambiguously labeled with a marker that is passed down to the progeny of each cell, revealing their subsequent contribution to the organism. Several sophisticated techniques have been developed (for review, see Buckingham and Meilhac, 2011; Kretzschmar and Watt, 2012), resulting in the elucidation of fate maps (e.g., Kimmel et al., 1990; Lawson et al., 1991), lineage relationships (e.g., Lescroart et al., 2010) and insights into organ growth and homeostasis by stem cells (e.g., Snippert et al., 2010; Centanin et al., 2011). A problem of clonal analysis is that the number of clones that can be followed in a single experiment is limited by the number of discernible labels. When the tissue of interest behaves cohesively, this problem is compensated because clones carrying the same label remain spatially distinct (e.g., Centanin et al., 2011). In contrast, cell migration and mixing generate ambiguity that can only be resolved if the population of interest is sampled using a large palette of labels. To this end, Livet et al. (2007) used site-specific recombination to achieve stochastic labeling with differently colored fluorescent proteins of neural and glial cells in the mouse cerebellum. The combinatorial colors generated by multiple, independently recombining transgene insertions further expanded the available color palette (Figure 2B). Their elegant approach, termed "Brainbow," has allowed the authors to distinguish the neurites of hundreds of cells (Figure 2C), which was instrumental to their computational circuit tracing analysis. This approach has since been successfully adopted to a variety of other systems and applications (e.g., Snippert et al., 2010; Hampel et al., 2011; Hadjieconomou et al., 2011).

Brainbow can be a tremendous help for automated cell tracking, but it comes at the cost of the requirement to record multiple channels, which may significantly impact temporal sampling. Not using LSFM, Mahou et al. (2012) achieved the simultaneous two-photon excitation of blue, green-yellow and red fluorophores in mouse cortex and chicken spinal cord that were clonally labeled with Brainbow cassettes. Additionally, they include third-harmonic generation during a short period of *Drosophila* gastrulation, resulting in four simultaneously recorded channels. Such a technique, implemented as LSFM, promises to be an elegant way to facilitate multicolor-aided tracking of many cells.

Visualizing Dynamic Gene Expression

Gene expression maps help to unravel gene function and regulatory networks. Of particular interest is the expression dynamics of key transcription factors with well-established roles in cell fate specification and that of other markers that unambiguously identify cell types. So far, much progress has been achieved using conventional imaging techniques. Large collections of information have been gathered using RNA in situ hybridization,

immunochemistry and transgenic expression reporters in stage-matched specimens (for review, see Lécuyer and Tomancak, 2008; de Boer et al., 2009). However, a detailed analysis of dynamic gene expression would benefit from higher temporal sampling and convenient 3D in toto coverage of large samples, which is provided by LSFM.

Recording gene expression in vivo requires faithful reporters. As the capabilities and sensitivity of microscopes increase, it becomes more important to consider reporter design. Transgenesis with large constructs such as bacterial artificial chromosomes (BACs), cosmids, and fosmids is widely used and has become further accessible with advances in high throughput recombination techniques (Poser et al., 2008). A general pitfall of this strategy is that positional effects may alter the readout of the reporter (Levis et al., 1985). This is particularly problematic when multiple reporters are to be assessed comparatively. A promising candidate to circumvent this problem is the PhiC31 phage integrase (Kuhstoss and Rao, 1991; Rausch and Lehmann, 1991), which catalyzes the site-specific integration of constructs up to the size of BACs into preestablished docking sites. It is successfully used in the *Drosophila* community (Groth et al., 2004; Ejsmont et al., 2009) and has additionally been applied in other model organisms (Belteki et al., 2003; Lister, 2010). Another approach to reveal physiological gene expression patterns is gene trap (Gossler et al., 1989). The strategy was initially designed for insertional mutagenesis, where it is used to prematurely terminate transcription, but has been extended to an approach that reports gene expression without necessarily being disruptive (Trinh le et al., 2011). In general, however, the most reliable strategy to ensure a faithful and quantitative readout is to genomically tag the gene of interest. Unfortunately, genomic homologous recombination is only available to few species such as mouse and *Drosophila*. However, recent reports of zinc-finger nuclease induced homologous recombination in mouse and rat embryos (Cui et al., 2011) raises expectations that this technology will be available for additional organisms.

Since only a few genes can be simultaneously labeled in a single specimen, it is desirable to computationally multiplex data from independent experiments. This requires an identification of corresponding regions or, ideally, even cells in different individuals. In *C. elegans*, researchers have solved this problem by utilizing the known invariant lineage. Libraries of gene expression reporters recorded with confocal microscopy provided quantitative expression readouts with cellular resolution. Combined with automated computational assignment of cell identity (Murray et al., 2008; Long et al., 2009; Qu et al., 2011), many genes were multiplexed at cellular resolution, yielding expression signatures for each cell. Data with such detail were used to analyze whether expression signatures correlate more with cell fate or mitotic lineage (Liu et al., 2009) and to perform very thorough quantitative descriptions of phenotypes induced by transcription factor deletions, including effects on gene expression in vivo (Boeck et al., 2011). Murray et al. (2012) reported a highly detailed analysis of the expression dynamics of 127 genes, predominantly transcription factors, with 1 min temporal sampling through the 350-cell stage of *C. elegans* (Figure 2D). The authors show that each cell's expression signature can be distinguished from that of most other cells during this stage of development. Within the diversity, overarching patterns correlating with tissue and position within the animal were observed as well.

In contrast to *C. elegans*, individual variability impacts on *Drosophila* embryogenesis. Fowlkes et al. (2008) derived a mean morphological template from hundreds of embryos at blastoderm stages. The pair-rule genes *even skipped* (*eve*) and *fushi tarazu* (*ftz*) were used as reference during image registration of the 3D expression patterns of more than 100 genes onto this prototype, with cellular resolution (Figure 3A). Furthermore, the authors performed a proof-of-concept analysis that confirms the usefulness of the strategy to detect gene regulatory interactions. It is unclear whether this approach can be adapted to other tissues and organisms. While there is continuous progress for the high-quality alignment of 3D gene expression domains (Tomer et al., 2010; Peng et al., 2011; Ronneberger et al., 2012), no similar analysis has been reported to date, in part because, until now, morphological prototypes of other tissues/species at cellular resolution have been unavailable.

The versatility of LSFM for the examination of dynamic gene expression in developing embryos is nicely exemplified by Reeves et al. (2012). The authors used two-photon LSFM to bidirectionally illuminate a transversal plane within a *Drosophila* embryo, resulting in a relatively uniform illumination that was essential to their detailed quantitative investigation. They reported a surprising temporal dynamic in the nuclear localization of the transcription factor Dorsal that patterns the dorsoventral axis during early *Drosophila* embryogenesis. This behavior is recapitulated by the expression dynamics of known Dorsal target genes. This study reconfirms that the Dorsal gradient is narrow, with a very shallow, almost linear, slope at the dorsal side of the embryo. The authors suggested time averaging to explain how the gradient can establish domains of graded gene expression dorsally, despite the low local changes in protein activity.

The aforementioned studies highlight the information on gene regulatory networks, cell type diversification, and cellular dynamics of lineage progression that is contained in quantitative gene expression data. So far, however, the complexity of most tissues and organisms has prohibited an analysis at absolute cellular resolution. One part of the problem is that gene expression dynamics occurs in the context of morphogenetic rearrangements. The combination of cell tracking and simultaneous monitoring of gene expression could provide important additional detail. To this end, both high spatial and high temporal sampling are required, a demand that is met by LSFM.

Cell Signaling and Cell Biological Approaches

Besides their internal genomic information, cells receive and process information from the environment and each other. Since most signaling pathways ultimately modulate gene expression, transcriptional reporters have proven extremely useful for the examination of pathway activity in various models (Laux et al., 2011; Chatterjee and Bohmann, 2012). Nonetheless, as discussed in the previous section, interpreting data obtained with transcriptional reporter transgenes requires careful consideration. Barolo (2006) reviewed the limitations and potential pitfalls of transcriptional Wnt reporters based on TCF-binding motifs. He outlined fundamental differences between classical reporter

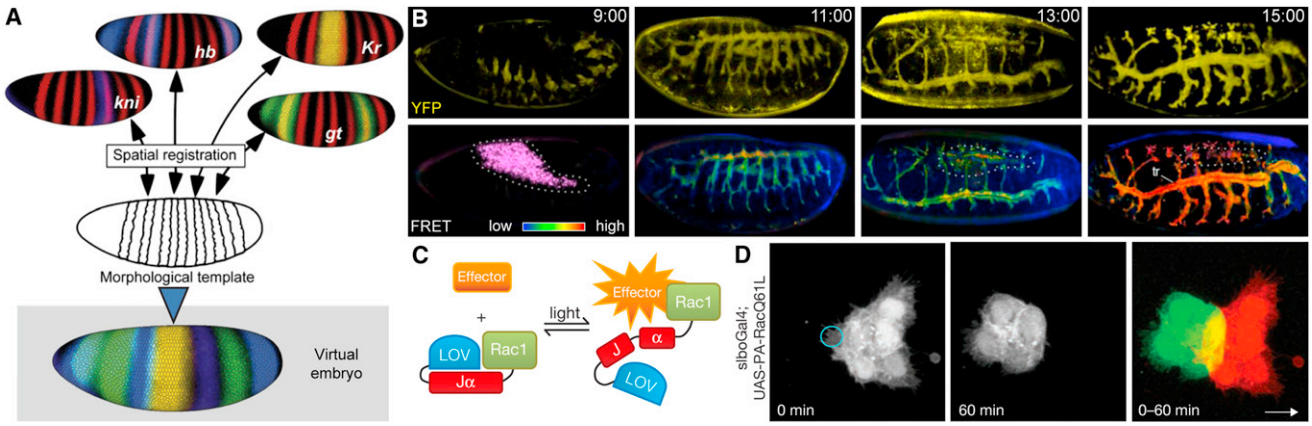

Figure 3. Recording Gene Expression, Cell Signaling, and Optogenetic Control

(A) *Drosophila* blastoderm gene expression atlas. Data from hundreds of individually imaged embryos were averaged into a composite virtual embryo. Top: each individual embryo was stained for nuclei, a common reference pair-rule gene (red, *even skipped* [*eve*] or *fushi tarazu* [*ftz*]) and a gene of interest (second color). Center: the reference gene guided spatial registration on to a morphological template. Bottom: correspondences across embryos, with averaged expression measurements, provided a model virtual embryo in which the expression of many genes can be analyzed. Adapted from Fowlkes et al. (2008) with permission from Elsevier.

(B) Time course (6 hr) of Cdc42 localization (yellow fluorescent protein [YFP]; top row) and activation (FRET efficiency, color bar; bottom row) in the *Drosophila* tracheal system. Reprinted from Kamiyama and Chiba (2009) with permission from AAAS.

(C and D) Optogenetic activation of Rac1 in *Drosophila* ovary border cells. (C) Schematic diagram showing the mechanism of photoactivatable Rac1 activation by light. Rac1 fused to the photoreactive light oxygen voltage (LOV) domain from phototropin (Christie et al., 1999; Harper et al., 2003) sterically blocks Rac1 interactions until light-induced unwinding of a helix linking LOV to Rac1. (D) Local photoactivation or photoinactivation of Rac in one cell affects the morphology and behavior of other cells in the group. Confocal images of border cell clusters before (0 min, green) and after (60 min, red) photoactivation. The area of laser treatment (circle) and the direction the border cells would normally migrate (white arrow) are indicated. Adapted from Wang et al. (2010) with permission from Macmillan Publishers Ltd.

and endogenous response genes and highlighted observed inconsistencies of independent, yet similarly constructed reporters. His conclusions are likely transferable to transcriptional reporters of other pathways. Finally, the molecular cascades underlying intracellular information processing are intrinsically fast, and the temporal domain of transcriptional reporters is a poor fit to both the dynamics of upstream signaling events and the recording speed obtainable with LSFM.

Capturing signaling events close to real time in vivo and on tissue, or even organism, scale is a formidable challenge that requires the recording of potentially large volumes with high speed. As described earlier, LSFM is very well suited to acquire such data. For instance, Holekamp et al. (2008) developed objective-coupled planar illumination microscopy, a variation of light sheet microscopy, since they required a high imaging speed and large field of view to simultaneously record the activity of hundreds of neurons in explanted mouse vomeronasal organ tissue. This approach was used to visualize the response of these neurons to stimulation with various pheromones (Turaga and Holy, 2012).

The visualization of molecular signaling events is not straightforward and requires in-depth knowledge of interaction partners, sites of posttranslational modifications, domain architecture, and structure of the components within a pathway. However, protein probes are invaluable tools because they enable exceptionally detailed insights into both temporal and spatial dynamics of signaling events. Many probes have been established and used with great success (Palmer et al., 2011). For instance, Michel et al. (2011) constructed a probe for bone morphogenetic protein (BMP) receptor activation. With this tool, the signaling dynamics between different cell populations in the *Drosophila* male germ stem cell domain could be dissected. Among other things, the authors showed that BMP receptor activation occurs exclusively at spatially confined sites where germ stem cells are in close contact with one of two BMP ligand producing candidate cell types.

The concentration, diffusion, and interactions of proteins in living cells can be quantified with fluorescence correlation spectroscopy (FCS) (Elson and Madge, 1974). Wohland et al. (2010) combined this method with LSFM and performed a proof-of-concept analysis with fluorescent microspheres injected into the bloodstream of a zebrafish embryo. Recently, light-sheet-based two-dimensional (2D)-FCS was used to map the mobility of GFP-tagged heterochromatin protein HP1-alpha in the nuclei of mammalian 3T3 fibroblasts (Capoulade et al., 2011).

Another generic principle for constructing sensors for protein-protein interactions and conformational changes is Förster resonance energy transfer (FRET). This approach has already proven useful for a variety of molecular reporters including activation of GTPase and growth factor receptors (Zhou et al., 2012). Notable applications in developmental biology include the observation of small GTPase dynamics during germ-cell migration in zebrafish (Kardash et al., 2010) and a spatiotemporal map of Cdc42 activity during *Drosophila* development (Figure 3B; Kamiyama and Chiba, 2009). One popular method to detect FRET is fluorescence lifetime imaging microscopy (FLIM, reviewed by Festy et al., 2007), which has been implemented as a proof-of-principle LSFM instrument (Greger et al., 2011). Techniques such as FCS and FRET have opened up the possibility to quantitatively study the molecular details underlying cell behavior and information processing. A combination with the strengths of LSFM appears to be an exciting way to study these processes with

high spatial and temporal resolution during extended periods of embryogenesis.

The implicit assumption for most of its applications is that microscopy is a tool for observation and that the effects of that observation on the specimen are essentially negligible. This can hold true to varying extents and, as described earlier, minimizing photoexposure is indeed a major motivation behind the development and application of LSFM. However, an increasing number of studies use microscopy specifically for both observation and experimental perturbation. A particular advantage of this approach is that interference can be performed with almost arbitrary spatiotemporal control. Laser microsurgery can been used to assess the forces acting on tissues during morphogenesis (e.g., Hutson et al., 2003) and a pulsed-laser-based microsurgery setup has been combined with LSFM to cut microtubules in cell-culture cells and to provoke laser injury induced immune responses in *Drosophila* embryos (Engelbrecht et al., 2007).

Optogenetic tools and effector molecules that are locked into photolabile cages provide additional ways to optically manipulate live specimens. For instance, focal uncaging of the neurotransmitter glutamate provided essential electrophysiological information for circuit mapping (e.g., Shao and Dudek, 2005). Arrenberg et al. (2010) expressed the light-activatable ion channels channelrhodopsin-2 and halorhodopsin in zebrafish cardiomyocytes to locate and control the pacemaker in the embryonic heart. The heartbeat rate was measured using a light sheet microscope that was outfitted with a digital micromirror device enabling precise control over the sites of ion channel activation. In another study using confocal microscopy, a photoactivatable Rac1 GTPase (Figure 3C) was used to modulate the migration of border cells in the *Drosophila* ovary in vivo (Wang et al., 2010). Focal, light-induced activation of Rac in a single cell sufficed to induce polarization in a large cluster of border cells, resulting in the establishment of an aberrant migration direction (Figure 3D). The optogenetic modulation of gene expression has been reported as well (Wang et al., 2012). Such tools may offer the intriguing opportunity to induce very subtle yet precisely defined phenotypes in a developing organism, e.g., by interfering with cell sorting, by delaying a specific migration event or by arbitrarily modulating gene expression. LSFM would allow the simultaneous observation of the effects, including long term, on the entire organism. The collection of optogenetic tools is steadily increasing (Rein and Deussing, 2012), and the publication of more tools and exciting applications in the context of a developing embryo seems just a matter of time.

Four-Dimensional Image Processing and Visualization

The ever-improving imaging instrumentation has transformed the way imaging is used from a tool for observations to a tool for measurements. This was made possible and is accompanied by the emergence of the field of bioimage informatics (Peng, 2008; Eliceiri et al., 2012). The manuscripts cited earlier highlight that the synergy between microscopy and image processing is further intensified in LSFM. For instance, the multiview acquisition that is central to data recording in many labs requires sophisticated image processing to merge the individual stacks recorded from different perspectives into a single volume with maximal data quality. More generally, with reported sustained data rates of, e.g., 350 Mb/s (Tomer et al., 2012), a single experiment can easily produce more data than is feasible to analyze manually. This problem is potentiated since LSFM is well suited for long-term imaging. Hence, automated data analysis is required to extract meaningful information from the images. The design and development of software that is capable of handling such data amounts with minimal user intervention requires a high level of in-depth computational expertise that is hard to come by in a typical developmental biology research group. Further use and adoption of LSFM may be facilitated by the agreement on and adherence to common standards and a culture of sharing in the community.

The large data amounts additionally require sophisticated visualization tools (Eliceiri et al., 2012; Scifer, http://www.scifer.info). So far, much progress was achieved in interactively handling the large 3D+t data sets. An additional challenge may arise due to the sheer number of processes that occur concurrently within a developing organism. For instance, when a large number of cells has been tracked, the resulting information is not necessarily informative per se, and an interpretation of the recorded events may be facilitated when cells are grouped by certain behavioral similarities (e.g., J. Fangerau et al., 2012, IEEE Symposium, conference). To date, this important aspect has not received much attention, since the scope of a typical single experiment was necessarily limited. As the technical issues associated with long-term in toto experiments are being overcome, we foresee an increasing demand for intelligent visualization tools that allow browsing the data at varying levels of complexity.

Conclusions

Confocal microscopy has evoked great enthusiasm, inspired ideas, and created the desire for increasingly advanced applications (Megason and Fraser, 2003). This enthusiasm is well justified, but some of the ideas have turned out to be beyond the capabilities of the confocal setup. The limitations of point scanning are particularly evident when observing larger specimens and requiring high temporal sampling over long time periods. In contrast, this application with high relevance for developmental biology is a strength of LSFM. Since its modern-day rediscovery, LSFM has developed at a fast pace. It is interesting to note that this development has been predominantly scientific and noncommercial; driven by increasing curiosity, demand, and applications in biology; and catalyzed by strong competition among the groups at the forefront of technology development. It will be exciting to see just how far light sheet microscopy can be pushed and which future applications it will inspire.

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